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Distribution of Iron-Containing Superoxide Dismutase in Vascular Plants[1,2]

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ABSTRACT

Superoxide dismutases (EC 1.15.1.1) in vascular plants representing different evolutionary levels were characterized using polyacrylamide gel electrophoresis. The three forms of the enzyme were distinguished from each other based on the following criteria: a) the Cu-Zn enzyme is sensitive to cyanide wherease the Fe and Mn enzymes are not; and b) the Cu-Zn and Fe enzymes are inhibited by H_2O_2 whereas the Mn enzyme is H_2O_2-resistant. Of the 43 plant families investigated, the Fe-containing superoxide dismutase was found in three families: Gingkoaceae, Nymphaceae, and Cruciferae.

Superoxide dismutases are metalloproteins found ubiquitously in all aerobic organisms. They serve a protective role against the deleterious effects of O_2 by catalyzing the disproportionation of the superoxide free radical ion to H_2O_2 according to the following equation (10):

$$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

Based upon metal content, three isozymes of superoxide dismutase have been identified. The Cu-Zn enzyme was the first isolated and characterized (21). It is found in vertebrates, land plants, and fungi. It has a mol wt of about 32,000, consists of two identical subunits, contains one atom of Cu and one of Zn per subunit and is distinguished by its inhibition by cyanide. The Mn enzyme has been found in bacteria as well as in the mitochondrial matrix of plants and animals (10, 13, 15, 25). This isozyme has a mol wt of 40,000 to 90,000 depending on source, a subunit number that varies from two to four, and a metal content of 1 to 4 atoms Mn per molecule (10, 20). The Mn enzyme is distinguished by its insensitivity to cyanide as well as H_2O_2. The third isozyme is an iron-containing protein. It has been found in procaryotes, has a mol wt of about 40,000, consists of two subunits, and has a metal content of 1 to 2 atoms Fe per molecule (10, 20). The Fe enzyme of SOD[3] is CN^--insensitive, but unlike the Mn enzyme, is H_2O_2-sensitive (5, 8).

Superoxide dismutases in photosynthetic organisms have been extensively studied (1–3, 11, 13, 19). Procaryotes and most eucaryotic algae lack the Cu-Zn SOD but contain either the Mn and/or Fe enzymes (3, 20). Land plants contain large amounts of the Cu-Zn enzyme plus an additional cyanide-resistant SOD which was assumed to be the Mn protein (3, 11, 17). The Fe enzyme was previously thought to be restricted to procaryotic organisms but has recently been purified from *Euglena gracilis* (14, 18) and, in our laboratory, from *Brassica campestris* (23).

Employing a modified staining technique for polyacrylamide gels that allowed us to visualize easily the three SOD isozymes in crude homogenates, we undertook a survey of a variety of vascular plants. In this paper, we report on other plant families found to contain the iron SOD.

MATERIALS AND METHODS

Mature leaves from native plants and cultivars were collected locally. Etiolated seedlings were grown from seed in a darkened chamber and harvested 7 to 10 days after sowing. Leaves or etiolated seedlings were ground in a Waring Blendor in ice-cold media consisting of 50 mM K-phosphate (pH 7.0), and 0.1% Triton X-100. The homogenate was squeezed through four layers of cheesecloth and centrifuged at 12,000*g* for 10 min. Leaf extracts were dialyzed overnight against 10 mM K-phosphate (pH 7.0), to remove small mol wt compounds which interfered with the activity stain for SOD on polyacrylamide gels.

Electrophoresis was performed on 7.5% acrylamide gels (9). The photochemical method of Beauchamp and Fridovich (6) was used to localize SOD activity on gels. The three types of SOD were distinguished by their different sensitivity to inhibitors (5, 8). Inhibitors (1 mM KCN or 2 mM H_2O_2) were added to the staining solution prior to addition to the gels. Gels were incubated in the staining solution for 45 min before exposure to light. Densitometric scans of gels were made with a Perkin-Elmer model 552-0800 integrating gel scanner. Spectrophotometric measurements of SOD activity were made by the method of McCord and Fridovich (21). Assays for SOD were routinely done in the presence of 10 μM KCN to inhibit Cyt oxidase. This low cyanide concentration was insufficient to inhibit the Cu-Zn enzyme.

RESULTS

The Cu-Zn enzyme could easily be distinguished from the other two isozymes by its sensitivity to cyanide. Asada *et al.* (5) and Britton *et al.* (8) demonstrated that the Cu-Zn enzyme from spinach and the Fe enzyme from *Plectonema* were inhibited by 0.5 mM H_2O_2, whereas the Mn enzyme was totally resistant to H_2O_2 at concentrations up to 5 mM. Densitometric scans of polyacrylamide gels of a crude extract of *B. campestris*, stained for SOD activity in the presence of CN^- and H_2O_2, are shown in Figure 1. Three major types of activity bands were observed without any inhibitors (trace A), two bands with CN^- (trace B), and one band with H_2O_2 (trace C). The CN^--sensitive, anodic group of bands (isozyme 3) are Cu-Zn isozymes. The middle band

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[3] Abbreviations: SOD, superoxide dismutase.

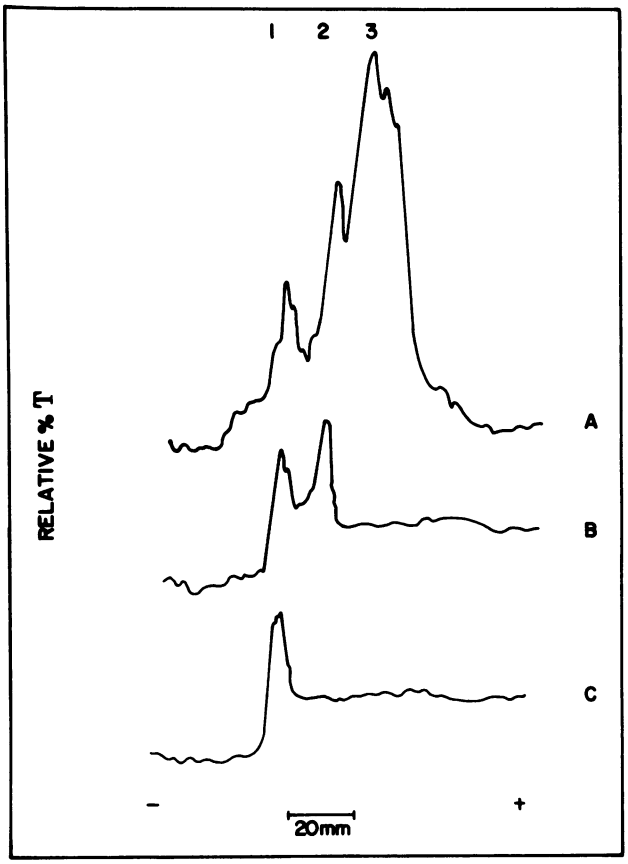

FIG. 1. Scans of gels of extract from mustard leaves stained for SOD activity. A: Standard staining mixture; B: Stained in the presence of 1 mM KCN; C: Stained in the presence of 2 mM H_2O_2.

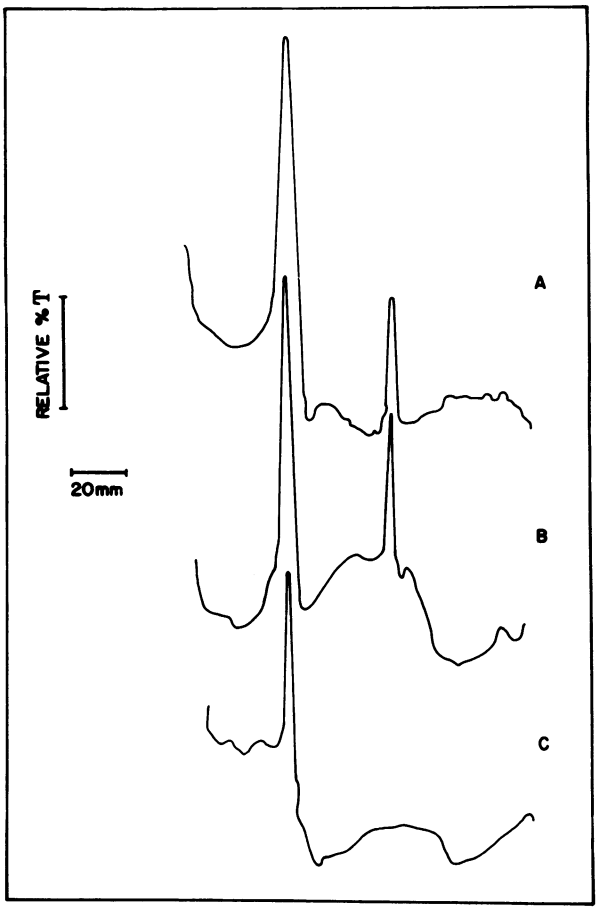

FIG. 2. Scans of gels of extract from *E. coli* stained for SOD activity. A: Standard staining mixture; B: Stained in the presence of 1 mM KCN; C: Stained in the presence of 2 mM H_2O_2.

(isozyme 2) corresponds in R_F value to the iron enzyme that we previously purified and characterized (23). The most cathodic band (isozyme 1) of activity which was CN^-- and H_2O_2-resistant represents the manganese SOD. Additional confirmation of the staining technique for identification of SOD isozymes was obtained from extracts of *Escherichia coli*. The Fe and Mn enzymes have been purified from this organism and their behavior on polyacrylamide gels as well as sensitivity to inhibitors has been documented (8, 15, 26). A gel scan of crude extracts from *E. coli* confirms that the more acidic Fe isozyme is sensitive to H_2O_2 whereas the basic Mn protein is not (Fig. 2). The recently devised method of distinguishing manganese SOD from iron SOD based upon the generation of apoenzyme by incubating the extract in guanidinium chloride (16), was unsuccessful with extracts from *B. campestris*. Our failure to distinguish the Fe enzyme by this method was probably due to the lability of the enzyme and the long duration of the procedure.

Table I is a composite of data obtained from the survey of vascular plants for various isozymes of SOD. Leaves or etiolated seedlings were used in the study because our initial observation of the Fe enzyme was in leaves and etiolated seedlings from *B. campestris* (24). The numbers in each column refer to the bands of activity (isoenzymes) observed. All the pteridophytes investigated contained both Cu-Zn and Mn SOD. The Fe enzyme was not detected. The primitive gymnosperm *Ginkgo biloba* L. exhibited two bands of activity corresponding to the Fe enzyme, yet no other gymnosperms examined were found to contain this isozyme.

None of the monocots investigated contained Fe SOD. The cyanide-resistant enzyme was the Mn protein. Dicot families containing Fe SOD were the Cruciferae and the aquatic Nymphaceae. All members of the Cruciferae surveyed contained Fe SOD activity bands that were easily distinguishable. The SOD patterns in the two species of Nymphaceae examined were unlike any other vascular plants investigated. As in green algae (3), these primitive dicots contained Fe and Mn SOD. The Cu-Zn enzyme was apparently absent.

Some plants did not exhibit a CN^--resistant activity on polyacrylamide gels (Table I). However, activity was found when SOD activity in most of these species was measured spectrophotometrically (Table II). The apparent absence of corresponding bands of activity on gels may be due to large amounts of pigmented materials that interfere with the light-mediated gel stain, thereby preventing use of high concentrations of extract for electrophoresis. Furthermore, cyanide-resistant activity, representing a small percentage of the total SOD activity, may be low in leaves (7). This latter point may explain the apparent absence of CN^--resistant activity in *Pilea pumila* on gels as well as in the quantitative assay.

DISCUSSION

The phylogenetic distribution of the three types of SOD has been widely studied and recently reviewed by Asada *et al.* (1) and McCord (20). The Mn enzyme is bound to the thylakoids of photosynthetic organisms (13, 14) and contained in the mitochondrial matrix (12). In eucaryotic algae, the Fe enzyme is found in the chloroplast stroma (14) but has been replaced by the Cu-Zn enzyme in organisms which evolved during the Cambrian period, *i.e.* land plants and fungi (1, 4, 12). In fact, the majority of vascular plants examined contained both the Cu-Zn and Mn enzymes but not the Fe enzyme. We have found a few exceptions which may

Table I. *Superoxide Dismutase Isozyme Activity Bands on Polyacrylamide Gels of Crude Extracts from Leaves or Etiolated Seedlings*

Extracts from designated species were homogenized, electrophoresed on polyacrylamide gels, and stained for SOD activity. Cu-Zn bands are those sensitive to 1 mM KCN. Fe bands are those resistant to 1 mM KCN yet inhibited by 1 mM H_2O_2. Mn bands are resistant to both KCN and H_2O_2. Each sample was run in triplicate.

	Species	No. of Activity Bands of Superoxide Dismutase		
		Cu-Zn	Fe	Mn
Pteridophyta				
Lycopodiaceae	*Lycopodium alopecuroides* L.	3	0	1
Equisetaceae	*Equisetum hyemale* L.	1	0	1
Pteridaceae	*Pteridium aquilinum* (L.) Kuhn.	1	0	1
Osmundaceae	*Osmunda regalis* L. var. *spectabilis* (Willd.) Gray	2	0	1
Spermatophyta-Gymnospermae				
Ginkgoaceae	*Ginkgo biloba* L.	3	2	0
Taxodiaceae	*Taxodium distichum* (L.) Rich.	3	0	0
Podocarpaceae	*Podocarpus Nagi* Makino	1	0	1
Taxaceae	*Taxus media Rehd.*	3	0	1
Cycadaceae	*Cycas revoluta* Thumb.	2	0	1
Angiospermae-Monocotyledoneae				
Liliaceae	*Lilium tigrinum* Ker.	3	0	1
Commelinaceae	*Commelina virginica* L.	2	0	1
Amaryllidaceae	*Allium Cepa* L.	2	0	1
Palmaceae	*Phoenix dactylifera* L.	3	0	1
Gramineae	*Lolium perenne* L.[a]	4	0	0
	Cynodon Dactylon (L.) Pers.	3	0	1
	Oryza sativa L.[a]	4	0	1
	Triticum aestivum L.[a]	2	0	0
	Sorghum vulgare Pers.[a]	3	0	1
Angiospermae-Dicotyledoneae				
Magnoliaceae	*Magnolia grandiflora* L.	4	0	0
Lauraceae	*Persea palustris* (Raf.) Sarg.	4	0	1
Berberidaceae	*Nandina domestica* Thunb.	2	0	1
Menispermaceae	*Cocculus carolinus* (L.) DC.	2	0	0
Nymphaceae	*Nymphaea odorata* Ait.	0	1	1
	Nuphar luteum (L.) Sibth. & Smith subsp. *macrophylum* (Small) Beal	0	4	1
Cruciferae	*Brassica campestris* L.	3	1	1
	Brassica Rapa L.[a]	1	1	1
	Brassica oleracea L.	1	1	1
	Raphanus sativus L.	1	1	1
	Rorippa sessiliflora (Nutt.) Hitchcock	2	1	1
Sarraceniaceae	*Sarracenia alata* (Wood) Wood	4	0	1
Malvaceae	*Hibiscus esculentus* L.	4	0	0
	Hibiscus militaris Cav.	4	0	0
Euphorbiaceae	*Croton monanthogynus* Michx.	2	0	0
Rutaceae	*Zanthoxylum Clava-Merculis* L.	3	0	1
Cyrillaceae	*Cyrilla racemiflora* L.	2	0	0
Aceraceae	*Acer rubrum* L.	2	0	1
Sapindaceae	*Cardiospermum Halicacabum* L.	1	0	1
Urticaceae	*Pilea pumila* (L.) Gray	2	0	0
Saururaceae	*Saururus cernuus* L.	1	0	1
Amaranthaceae	*Iresine rhizomatosa* Standl.	3	0	0
Portulacaceae	*Portulaca grandiflora* Hook.	3	0	0
Symplocaceae	*Symplocos tinctoria* (L.) L'Her.	3	0	0
Solonaceae	*Lycopersicon esculentum* Mill.	2	0	1
	Physalis angulata L.	2	0	2
Verbenaceae	*Phyla lanceolata* (Michx.) Greene	2	0	0
Labiateae	*Ocimum basilicum* L.	2	0	1
	Pycnanthemum albescens T. & G.	1	0	0
Scrophulariaceae	*Scoparia dulcis* L.	2	0	1
Pedaliaceae	*Sesamum indicum* L.	3	0	0
Acanthaceae	*Dicliptera brachiata* (Pursh) Spreng.	2	0	0
	Hygrophilia lacustris (Schlecht) Nees	4	0	0
	Justicia lanceolata (Chapm.) Small	3	0	1
Leguminosae	*Glycine max* (L.) Merrill[a]	6	0	1
	Phaseolus vulgaris L.	2	0	1
Lythrareae	*Ammannia coccinea* Rottb.	1	0	1
Umbelliferae	*Apium graveolens* L. var. *dulce* Pers.	2	0	1
	Daucus Carota L. var. *sativa* DC.	2	0	0
	Petroselinum crispum (Mill.) Mansfield	2	0	1
Cucurbitaceae	*Cucurbita Pepo* L.[a]	3	0	2
	Cucumis sativus L.[a]	3	0	2
	Cucumis Melo L.[a]	4	0	2
	Cayoponia quinqueloba (Raf.) Skinners	3	0	0
	Citrullus vulgaris Schrad.	3	0	2
Compositae	*Tagetes patula* L.	2	0	0

[a] Etiolated seedlings used for assay.

Table II. *Cyanide-Resistant SOD Activity Measured Spectrophotometrically*

Leaf extracts were prepared and SOD activity assayed by the Cyt *c* method as described. SOD activity in the presence of 1 mM KCN was compared to that in the presence of 10 μM KCN.

Species	Cyanide-Resistant SOD Activity
	%
Pilea pumila (L.) Gray	0
Phyla lanceolata (Michx.) Greene	10
Sesamum indicum L.	20
Dicliptera brachiata (Pursh) Spreng.	13
Cayoponia quinqueloba (Raf.) Shinners	13
Nuphar luteum (L.) Sibth. & Smith	99

necessitate modification of existing theories of evolution of SOD. There appear to be no phylogenetic relationships among the families of vascular plants containing the Fe enzyme. The appearance of the Fe enzyme in isolated plant families might be accounted for by independent occurrences of gene transfer from bacteria or algae to eucaryotic, vascular plants. A case of transfer of the Cu-Zn SOD gene from a host fish to the symbiotic bacterium, *Photobacterium leiognathi* (22), has been reported (J. P. Martin, Jr., and I. Fridovich, manuscript submitted).

An alternate explanation for the random occurrence of the Fe enzyme in vascular plants is possible. Upon appearance of the Cu-Zn enzyme in the ancestor of vascular plants, modifications may have occurred in the Fe enzyme causing loss of dismutase activity and acquisition of a differing enzymic function. This would result in vascular plants having an iron-containing enzyme present but not functioning as a SOD. Families having iron SOD activity would represent cases in which the protein had been modified such that SOD activity had been reacquired. We are planning to test the hypothesis (protein with lost SOD activity) by preparing antibody to Fe SOD and testing for cross-reactivity in plants lacking iron dismutase function.

A third possibility is that the gene for the Fe enzyme is present in all eucaryotic plants but not expressed. Environmental pressures could have resulted in the selection of a modified controlling region arising by mutation and allowing once more for expression of the enzyme in Gingkoaceae, Cruciferae, and Nymphaceae.

The puzzling situation in the Nymphaceae where the Cu-Zn SOD appears to be absent will also require further investigation, including cross-reactivity studies with antibody to the plant Cu-Zn enzyme. Perhaps the apparent lack of the Cu-Zn isozyme in this bottom-rooted aquatic plant family is a reflection of an environment poor in Cu, inasmuch as in an anaerobic environment, most Cu would be bound as the insoluble copper sulfide.

Our study, while lengthy, was not exhaustive. The appearance of the Fe enzyme in isolated plant families may portend its existence in others which we have been unable to examine.

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